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NOTE

Isolation and characterization of novel microsatellite markers for a globally distributed invasive ant *Paratrechina longicornis* (Hymenoptera: Formicidae)

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Abstract. The longhorn crazy ant, *Paratrechina longicornis* (Latreille), is a ubiquitous agricultural and urban pest that has invaded most tropical and subtropical regions. Although *P. longicornis* has been found worldwide for more than a century, the genetic structure, origin, and invasion history of this species have not yet been extensively studied, partially because of the limited number of genetic markers currently available. In the present study, we developed 36 polymorphic microsatellite markers for *P. longicornis* and characterized these markers by genotyping *P. longicornis* workers from 74 colonies in East and Southeast Asia. All loci were polymorphic, with the number of alleles per locus ranging from 3 to 18 (8.5 on average). Extremely high levels of heterozygosity were found in all populations, suggesting that workers are invariably produced from the mating of divergent queen and male lineages. Queens and males possess non-overlapping allele size ranges at 18 loci, indicating the potential resolving power of the subset of markers in inferring the history of queen and male lineages. Genetic differentiation among three studied populations was low yet significant and may likely reflect their close association with human activities. Overall, the new microsatellite markers developed in the present study serve as a practical tool to reconstruct routes of invasion and assess the population genetics of this invasive ant.

INTRODUCTION

Biological invasions are a major threat to biodiversity and economic activity (Schmitz & Simberloff, 1997; Pimentel et al., 2000; Occhipinti-Ambrogi & Savini, 2003). While a considerable amount of effort has been devoted to prevent, control, and eradicate invasive species worldwide, management strategies designed to mitigate their negative impacts partially rely on reconstructions of invasion routes; these enable the immediate source to be identified and thus facilitate the design of strategies for controlling (e.g., biocontrol agent) or preventing invasions (Hulme, 2009; Wilson et al., 2009; Estoup & Guillemaud, 2010). Reconstructing routes of invasion based on historical observation data is challenging since the data are often sparse and incomplete (Estoup & Guillemaud, 2010); however, this limitation can now be overcome by implementing DNA-based molecular tools (e.g., molecular markers) that give rise to much higher resolution when inferring potential invasive pathways. Molecular markers such as microsatellite markers and mitochondrial DNA (mtDNA) sequences have been widely applied to identify migration pathways, quantify gene flow among populations across spatial scales, estimate admixture between populations from different origins, and can be further used to reconstruct the invasive routs of an alien species (Lawson Handley et al., 2011). For example, the global invasion histories of the red imported fire ant, *Solenopsis invicta*, and the tropical fire ant, *S. geminata*, were both revealed by using mtDNA and an extensive number of microsatellite markers (Ascunce et al., 2011; Gotzek et al., 2015). Recent developments in sequencing technologies have allowed the quick and economic development of a large number of molecular markers for non-model species (Yang et al., 2015). Microsatellites have emerged

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as the markers of choice for high-resolution population analysis because of the advantages of high variability, easy access, and low cost (Guichoux et al., 2011).

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802), is regarded as a significant invasive species due to its ecological impacts (Wetterer, 2008). The native range of this invasive species and its invasion history, however, remain controversial (Wetterer, 2008; LaPolla & Fisher, 2014). A previous study reported that colonies of *P. longicornis* from Bangkok, Thailand display a remarkable genetic system, whereby workers are produced by sexual reproduction, whereas queens are clones of their mothers and males are clones of their fathers (Pearcy et al., 2011). Under this system, workers carry high levels of heterozygosity as they are produced from divergent queen and male clones. As a consequence, the spatial pattern of genetic variation may be biased if analyzing the worker genotype alone due to the strong sex-associated structure between their male- and female-derived genomes. To overcome this, sexuals (i.e. male, queen and/or daughter queen) should be used instead (e.g., Fournier et al., 2005; Kuhn et al., 2017) or, where sexuals are not available, male and queen lineages can be inferred based on worker genotype (e.g., Darras et al., 2014). *Paratrechina longicornis* sexuals are, however, difficult to find in the field, being located deep within the nest in cavities of concrete objects with narrow crevices, and produced only during the warm, rainy months (Trager, 1984; Tseng, pers. observ.). This limitation, therefore, highlights the need to develop microsatellite markers that can infer male and queen lineages from the worker genotype. We note that of the 15 previously published microsatellite markers (Molecular Ecology Resources Primer Development Consortium et al., 2011), 11 display the potential to distinguish queen and male alleles from worker genotypes (Pearcy et al., 2011). To increase the power and resolution of infering the invasive history of *P. longicornis*, we developed a new set of polymorphic microsatellite makers and characterized the novel markers using worker and sexual samples from three geographical regions in East and Southeast Asia.

MATERIAL AND METHODS

Development of microsatellite markers

DNA libraries were prepared from genomic DNA of two *P. longicornis* queens from Thailand using a TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequencing on an Illumina HiSeq 2000. De novo genome assembling was carried out by Kuora Co., Ltd (Taipei, Taiwan) following the procedure described below. Trimming and error-correction were performed with Trimmomatic (Bolger et al., 2014) and BBMap software (Bushnell, 2015). Error-corrected reads were assembled using Platanus (Kajitani et al., 2014). The draft contigs were then screened for microsatellite loci containing 10 or more dinucleotide repeats using MSATCOMMANDER (Faircloth, 2008). A total of 65 potential microsatellite loci were selected, and primer pairs were designed using the Primer3 program (Rozen & Skaletsky, 1999) embedded in MSATCOMMANDER with default settings (product sizes ranging from 150 to 350 bp).

The 65 potential microsatellite loci were screened for positive PCR amplification using agarose gel electrophoresis. PCR reactions contained a total volume of 20 µl, composed of 10 µl of EmeraldAmp® MAX PCR Master Mix (TaKaRa, Otsu, Shiga, Japan), 1 µl of 10 µM primer pairs, 8 µl of ddH_2O and 1 µl of genomic DNA from one adult male (50 to 100 ng). The PCR conditions were as follows: initial denaturation at 94°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (40 s), with a final extension phase at 72°C (7 min). All obtained PCR products underwent gel electrophoresis; those that yielded a single band with the expected size were sequenced by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI-3730 autosequencer to confirm if they corresponded to the expected microsatellite loci. The nucleotide sequences of confirmed microsatellites were deposited in NCBI GenBank (accession nos. KY912037–KY912074) (Table 1).

Sampling, DNA extraction, and microsatellite genotyping

Paratrechina longicornis samples were collected between 2012 and 2015 from 74 colonies in three geographical regions, namely Taiwan (Taiwan island, 27 colonies), Thailand (central Thailand, 29 colonies), and Okinawa (Okinawa island, Japan, 18 colonies). The distance between each sampled colony was at least 100 m. One worker per colony was used for subsequent population genetic analyses. In addition, 8 queens (Taiwan, 2 colonies; Thailand, 2 colonies) and 5 males (Taiwan, 3 colonies) were sampled and used in genetic analyses. Genomic DNA was extracted using the Gentra Puregene Cell and Tissue Kit (Qiagen, Maryland, USA) according to the manufacturer's instructions and stored at –20°C until use.

In order to genotype all individual ants in an economic manner, we performed multiplex PCR reactions with fluorescently labeled universal primers following the strategy described in Blacket et al. (2012). Four fluorescent labeled universal primers and modified locus-specific primers with a 5' universal primer sequence tail were used. Five to six loci were amplified per multiplex reaction. PCR reactions contained a total volume of 20 µl, composed of 10 µl of EmeraldAmp® MAX PCR Master Mix (TaKaRa, Otsu, Shiga, Japan), 1.5 µl of 10 µM primer pairs, 7.5 µl of ddH_2O and 1 µl of genomic DNA from the ant sample (50 to 100 ng). The PCR conditions were as follows: initial denaturation at 94°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s), with a final extension phase at 72°C (30 min). The resulting PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker program (version 2.4.0, SoftGenetics LLC) was used to visualize and score alleles.

Characterization of microsatellite loci

Summary statistics of novel microsatellite markers including the number of alleles (Na), Shannon's information index (*I*), and observed heterozygosity (*Ho*) were calculated using GenAlEx 6.5 software (Peakall & Smouse, 2006). Regional genetic differentiation, as expressed by Wright's F_{ST} (F_{ST}), Jost's estimate of differentiation (*Dest*), and Hedrick's standardized G_{ST} for small number of populations (G''_{ST}), was estimated using GenAlEx 6.5 software (Peakall & Smouse, 2006).

RESULTS

Among 65 primer sets tested, 36 succeeded in amplification and showed polymorphisms (Table 1). A total of 305 alleles were amplified from the 36 loci based on the 74 genotyped worker individuals. The number of alleles per locus ranged from 3 to 18, averaging 8.5 alleles per locus for the worker dataset (Table 1). The presence of null alleles was unlikely as the vast majority of workers were heterozygous (Table 2). All 36 loci were successfully amplified from queen and male samples, and the number of alleles per locus ranged from 1 to 6 in queens and 1 to 3 in males (Table 1). Among the 36 loci, queens and males had non-overlapping allele size ranges at 18 loci (Table 1, highlighted in bold).

From the 305 alleles observed in the worker dataset, 40, 45, and 2 private alleles (i.e., the number of alleles unique to a single population) were found in Taiwan, Thailand, and Okinawa populations, respectively. The frequencies of private alleles were generally low, with average frequencies of 0.026, 0.033, and 0.083

Table 1. Summary of general information for the 36 polymorphic microsatellite loci isolated from *Paratrechina longicornis*. (Na-W – number of alleles in workers; Na-Q – number of alleles in queens; Na-M – number of alleles in males; Ta – annealing temperature). The loci which queens and males have non-overlapping allele size ranges are highlighted in bold.

Locus	Repeat motif	Primer sequences (5'−3')	Na-W	Na-Q	Na-M	Ta (°C)	Size range (bp)	Accession no.
Prl102	(CT)^12	F: TCCAACTGACCCGGAAGAC R: CGTACGGAATCGTGCGAAG	5	2	2	58	159–171	KY912037
Prl104	(AG)^15	F: GAGAGGGAACCCTGCTTCG R: TCTGCCTGGTTTAGCCCTC	13	5	2	58	263–291	KY912038
Prl106	(AT)^17	F: CTCATCGACCCTTTGACGG R: ACTGGTAAGTCCACTCCGC	13	4	3	58	286–320	KY912039
Prl107	(AT)^10	F: TCTCTGCAGCTGTGTCAGG R: CGCAATTAGCGTCTCCGC	8	1	3	58	294–330	KY912040
Prl109	(CT)^12	F: CAGTCGCAACAATGGCGG R: TGACGAAAGCACCCGTAGG	5	2	2	58	178–186	KY912041
Prl110	(CT)^15	F: CGTTATCCGTTCGTCACCG R: GTGTCCGATGCAAATCCCG	14	1	3	58	179–231	KY912042
Prl111	(AG)^13	F: AGCTGTCTGATTTCGTCGC R: AACGCCTTTAATCCGTCGC	14	4	3	58	277–317	KY912043
Prl113	(AT)^10	F: ATACACATTAGTGCATCCAACC R: TTCGGCGTTCGTGAACAAG	6	2	2	58	296–310	KY912044
Prl118	(AG)^16	F: ACAGGAAGTCGCGGAGATG R: AATGCGGTGGTCAAAGTGC	8	2	2	58	255–279	KY912045
Prl119	(AT)^13	F: ACAACTAATCGCCCGTAGC R: TGGATCGTGAGATTTCCGTTTAG	5	1	3	58	288–306	KY912046
Prl120	(AG)^17	F: CGCATGTGAATGTAAACGATGG R: CAGCTTGCGGTTCAAGGTC	18	6	1	58	297–341	KY912047
Prl121	(CT)^10	F: TAGTGCTGGATGCAGGGTG R: ACGGCGTAGTACCTTCTGC	5	2	1	58	307–315	KY912048
Prl123	(AG)^12	F: ACCGCAGCGTTAATTGC R: GTCTCCGGACCCATTCTCG	6	1	2	58	209–225	KY912049
Prl125	(CT)^10	F: AACACGGATGATTGCATGTC R: GCCGTGATACGAACTTCCAC	7	4	1	58	281–301	KY912050
Prl126	(AT)^11	F: AAGAACTGCAAGAGTGCGG R: GCACGTCCCGAGAAACATC	6	2	2	58	301–317	KY912051
Prl127	(AG)^12	F: AGCTTCCCGTACTTACACG R: TGCAGAAAGTATGTCGCGATG	4	2	1	58	315–325	KY912052
Prl128	(AT)^15	F: AAATTCGTCATGTTCCAGATCC R: CAGCTGGCAAGGCATGAAC	10	3	2	58	312–340	KY912053
Prl130	(CT)^11	F: GCACGCGGAAGCAATTAAC R: GGACGCGTTGGAAAGTTCG	3	1	2	58	221–225	KY912054
Prl132	(CT)^14	F: GATGGCGGAAATACCGGAG R: TCGTTGACTTTACGTGTCGC	5	1	2	58	283–291	KY912055
Prl136	(AT)^14	F: TTGACACAGAAGGCATTTCG R: AGACGGGAGGAAATATCACGG	9	1	3	58	212–244	KY912056
Prl137	(AG)^20	F: CTTTACGTCCGCCGTTTCC R: CATACCTCGCATGGTACGC	11	2	2	58	215–243	KY912057
Prl138	(AG)^17	F: TAGACGGATTCTCCACGGC R: TCTTCGACGGAGGTTCGTG	8	1	2	58	206–230	KY912058
Prl139	(CT)^20	F: TCGATTGACCCGAATCCCG R: TTGTCAAGCCACGAGCATC	12	3	2	58	282–306	KY912059
Prl141	(AT)^18	F: CTGCGCAAATTGTTCTGCC R: TCCATCGTAGGAAGTCGGTC	11	2	3	58	313–353	KY912060
Prl143	(AG)^10	F: GGCTCGGAATAGCTTCCAC R: GTCCCGAGCGCAGTTTATG	6	2	2	58	220–234	KY912061
Prl144	(CT)^10	F: GACGGGTATCGGAACTTTGC R: ACCGCGTTATTTCCGGTTG	10	3	1	58	232–276	KY912062
Prl149	(AG)^12	F: AGACCATGGATCACTCCGC R: TCCGTACATTAATATTCTGCAGTTG	7	2	3	58	345–363	KY912063
Prl150	(AG)^11	F: TCAACCGTAGCATGTGTCTTC R: TCGACATTCTTCCAATTTCGTG	4	1	1	58	240–246	KY912064
Prl152	(GT)^16	F: TCACTATGCGACATCAACTATCG R: CGCGTAAATAAACACGCTTCC	8	2	2	58	249–273	KY912066
Prl155	(GT)^10	F: ATCAGCCAAAGGAATTAGCAC R: ACACCTCACATCTCTTGAATGG	3	1	1	58	347–359	KY912068
Prl156	(AT)^17	F: CTCAGCAGCGAGTTGTTCG R: TGCGGCTTTATATCGGAGC	12	2	3	58	346–376	KY912069
Prl158	(GT)^11	F: CTGCTTGTTCACATGTTCGC R: CGTGCTCGCATGTATGATTTC	6	2	1	58	266–294	KY912070
Prl161	(AG)^12	F: CCCAATGGCGCAGATAACG R: ACAGATTTAAAGCCAGCGCC	7	1	2	58	355–385	KY912071
Prl162	(AT)^13	F: GCGCGTAATCGACCAACTC R: GTTTCAAGGGCTCCTTCGC	11	2	3	58	347–379	KY912072
Prl165	(AG)^18	F: GATTGCTTCCTCGCGCTAC R: TTCTCTGTGCTGCGAAACG	8	2	2	58	283–299	KY912073
Prl166	(AG)^16	F: ACGTGGAATTCGTTTCGGC R: GAAGCCCATTCGCCCATTC	17	4	3	58	283–331	KY912074

Table 2. Genetic diversity across the 36 polymorphic microsatellite loci in *Paratrechina longicornis* workers from Thailand, Taiwan and Okinawa (N – sample size; Na – number of alleles; *I* – Shannon's information index; *Ho* – observed heterozygosity).

Locus	Taiwan				Thailand				Okinawa			
	N	Na	*I*	*Ho*	N	Na	*I*	*Ho*	N	Na	*I*	*Ho*
Prl102	27	4	1.212	1	29	4	1.087	0.931	18	3	0.781	0.778
Prl104	26	8	1.849	1	29	12	2.036	1	17	7	1.6	1
Prl106	27	12	2.09	0.889	29	12	2.234	1	18	8	1.842	0.944
Prl107	25	6	1.263	1	28	5	1.208	1	17	3	0.966	1
Prl109	27	5	1.304	0.815	29	5	1.304	0.862	18	5	1.2	0.778
Prl110	27	10	1.69	0.963	29	9	1.317	1	18	3	0.958	1
Prl111	26	8	1.802	1	29	11	1.842	1	18	7	1.604	1
Prl113	27	5	1.329	1	29	6	1.414	1	17	4	1.212	1
Prl118	27	8	1.505	1	29	6	1.391	1	18	5	1.307	1
Prl119	24	5	1.052	1	29	4	1.102	1	16	3	1.04	1
Prl120	27	12	2.174	1	29	14	2.099	1	18	7	1.483	1
Prl121	27	5	1.017	0.741	29	5	0.893	0.517	18	4	0.877	0.667
Prl123	27	6	1.289	0.926	26	5	1.172	0.962	16	3	0.769	0.75
Prl125	27	7	1.705	0.926	28	7	1.66	0.964	18	7	1.76	0.889
Prl126	27	6	1.363	0.889	29	5	1.092	0.931	18	2	0.692	0.944
Prl127	27	4	0.979	1	29	4	0.892	1	18	3	0.8	1
Prl128	27	10	1.858	1	29	8	1.703	1	17	6	1.501	1
Prl130	27	2	0.386	0.259	29	3	0.379	0.207	18	2	0.349	0.222
Prl132	27	4	1.039	0.889	29	4	1.074	0.931	18	3	0.949	0.944
Prl136	27	6	1.269	1	29	7	1.375	1	18	4	1.211	1
Prl137	27	8	1.647	1	29	10	1.484	1	18	6	1.388	1
Prl138	27	7	1.182	0.704	29	7	1.383	0.966	18	5	1.286	1
Prl139	27	9	1.981	0.926	29	11	2.051	1	18	8	1.771	0.889
Prl141	27	9	1.475	1	29	9	1.605	1	18	6	1.393	1
Prl143	27	6	1.109	0.556	29	5	0.825	0.379	18	4	0.693	0.389
Prl144	27	9	1.854	0.963	29	8	1.719	1	17	6	1.558	0.941
Prl149	25	7	1.723	1	29	6	1.623	0.931	18	6	1.563	0.944
Prl150	27	3	0.184	0.074	29	3	0.546	0.345	18	3	0.411	0.222
Prl152	27	8	1.598	0.889	29	5	1.258	0.793	17	6	1.589	0.941
Prl155	26	3	1.034	1	27	3	1.031	1	18	3	1.037	1
Prl156	27	10	1.661	0.926	29	10	1.808	0.862	18	7	1.353	0.778
Prl158	27	5	1.087	1	28	5	1.117	1	18	4	0.906	1
Prl161	26	6	1.265	0.962	29	6	1.295	0.862	18	4	0.932	0.667
Prl162	26	8	1.669	1	29	10	1.623	1	18	6	1.415	1
Prl165	27	6	1.392	1	28	8	1.551	1	17	4	1.151	1
Prl166	27	13	2.322	1	29	13	2.06	1	18	11	2.183	1
Mean (SD)	27(0.7)	7(2.6)	1.427(0.451)	0.897(0.206)	29(0.6)	7(3.0)	1.396(0.434)	0.901(0.203)	18(0.6)	5(2.0)	1.209(0.413)	0.880(0.210)

for *P. longicornis* in Taiwan, Thailand, and Okinawa, respectively. The number of alleles (Na), Shannon's information index (*I*), observed heterozygosity (*Ho*), and expected heterozygosity (*He*) for each region are listed in Table 2. The microsatellite polymorphism and genetic diversity, as expressed by the average number of alleles per locus and average Shannon's information index, were generally higher in *P. longicornis* in Taiwan and Thailand than in Okinawa (Table 2). *Paratrechina longicornis* in all regions displayed remarkably high levels of observed heterozygosity, with average values of 0.897, 0.901, and 0.880 for ants in Taiwan, Thailand and Okinawa, respectively (Table 2). Pairwise F_{ST} values were very low in all pairwise population comparisons, ranging from 0.016 to 0.020 (Table 3). Similar results were found for *Dest* and G''_{ST} yet genetic differentiation between regions were significant (*Dest* = 0.032–0.048; G''_{ST} = 0.048–0.070; Table 3). Overall, our data reveal a high degree of genetic variability, and thus highlight the substantial potential of these newly developed markers on population genetic studies.

Table 3. Pairwise genetic differentiation among the three studied *Paratrechina longicornis* populations.

Population pair	F_{ST}	*Dest*	G''_{ST}
Thailand vs. Taiwan	0.018**	0.048**	0.070**
Taiwan vs. Okinawa	0.020**	0.047**	0.069**
Thailand vs. Okinawa	0.016*	0.032*	0.048*

Significance was tested using 999 permutations for pairwise F_{ST}, *Dest*, and G''_{ST} (*P < 0.05; **P < 0.01).

DISCUSSION

In the present study, we developed a set of 36 microsatellite markers for the longhorn crazy ant *P. longicornis*. Descriptive statistics of the 36 microsatellite loci across all studied regions indicate that these loci are sufficiently polymorphic to conduct population genetic studies on this invasive ant. Among the 36 loci, queens and males had non-overlapping allele size ranges at 18 loci, implying these loci could be an ideal tool to infer genetic lineages of queens and males from worker data.

Our genetic analyses reveal a low yet significant level of genetic differentiation in *P. longicornis* among the three studied regions. One explanation for this pattern is that these three regions were colonized by genetically similar source populations and the time since introduction may have not been sufficient for drift or local adaptation to produce a measurable level of genetic differentiation. It is also likely that ongoing gene flow associated with extensive international commerce activities may have erased the signature of genetic differentiation among the three regions, given that *P. longicornis* is one of the most common ants found or intercepted on human-associated means of transport (Weber, 1939; Lester, 2005; Wetterer, 2008).

The levels of heterozygosity across the 36 loci were extremely high in workers of all studied regions. The high heterozygosity most likely results from the unusual reproductive mode of this species whereby workers are produced from hybrid mating from divergent queen and male clones. Our study suggests that this system is likely widespread in Asia and might be linked to the invasion success of *P. longicornis* as it acts as an adaptive trait to relax the costs associated with inbreeding (Pearcy et al., 2011).

In conclusion, we have developed 36 high-quality microsatellite markers for *P. longicornis*. These novel markers, combined with 15 previously published microsatellite markers (Molecular Ecology Resources Primer Development Consortium et al., 2011), potentially allow us to have higher resolving power in inferring the routes of introduction of *P. longicornis* and examining the population structure of this ant at a variety of geographical scales. These data, if obtained, would serve as baseline information for developing an effective control scheme or formulating appropriate quarantine procedures on *P. longicornis*.

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COMPLIANCE WITH ETHICAL STANDARDS. CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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